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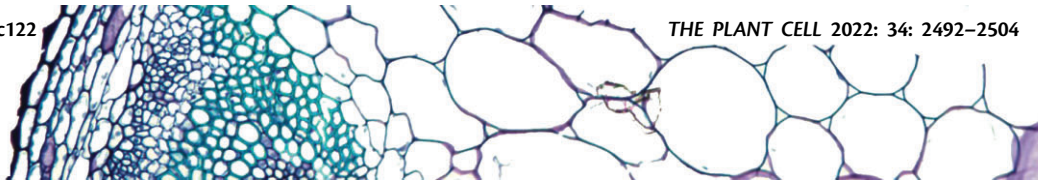

Review

Plant telomere biology: The green solution to the end-replication problem

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Abstract

Telomere maintenance is a fundamental cellular process conserved across all eukaryotic lineages. Although plants and animals diverged over 1.5 billion years ago, lessons learned from plants continue to push the boundaries of science, revealing detailed molecular mechanisms in telomere biology with broad implications for human health, aging biology, and stress responses. Recent studies of plant telomeres have unveiled unexpected divergence in telomere sequence and architecture, and the proteins that engage telomeric DNA and telomerase. The discovery of telomerase RNA components in the plant kingdom and some algae groups revealed new insight into the divergent evolution and the universal core of telomerase across major eukaryotic kingdoms. In addition, resources cataloging the abundant natural variation in *Arabidopsis thaliana*, maize (*Zea mays*), and other plants are providing unparalleled opportunities to understand the genetic networks that govern telomere length polymorphism and, as a result, are uncovering unanticipated crosstalk between telomeres, environmental factors, organismal fitness, and plant physiology. Here we recap current advances in plant telomere biology and put this field in perspective relative to telomere and telomerase research in other eukaryotic lineages.

Introduction

Mendel launched a revolution in biology with his pioneering insights into genetics, paving the way for groundbreaking discoveries in chromosome biology for more than a century. In this long tradition, Barbara McClintock's studies in maize (*Zea mays*) some 80 years ago revealed the critical function of telomeres (McClintock, 1939, 1941). Telomeres are now recognized as key components of the genome. They not only link the architecture of the DNA terminus with proliferative capacity and cellular aging, but also serve as sentinels for intrinsic and extrinsic stressors (Barnes et al., 2019; Lin and Epel, 2022).

Telomeres consist of simple G-rich DNA repeats that typically terminate in a single-stranded 3′-overhang. Through their unique structure and dedicated proteins, telomeres distinguish the ends of chromosomes from DNA damage, thereby solving the "end-protection" problem. End protection requires sequestration of chromosome termini to avert activation of a DNA damage response. In most organisms, chromosome end protection is accomplished via (1) formation of terminal t-loops in the DNA with the 3′-overhang tucking back into the duplex region and (2) engagement of telomere proteins, usually the shelterin or Conserved Telomere maintenance Component 1 (CTC1)/(Cell Division Cycle 13 (Cdc13), Suppressor of cdc ThirteeN (STN1), and

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TElomeric pathway with stN1 (TEN1) (CST) complexes. Together, the t-loop and telomere-associated proteins ensure that chromosome termini do not solicit the DNA damage checkpoint machinery, leading to extensive DNA degradation or end-to-end chromosome fusion.

Telomeres also solve the "end-replication problem" by facilitating the complete replication of chromosome ends through coordination of DNA replication machinery and telomerase. It was foreseen in the 1970s that conventional DNA polymerases are incapable of fully replicating the very termini of linear chromosomes (Watson, 1972; Olovnikov, 1973), causing a gradual attrition of telomere DNA over cell divisions that would ultimately result in chromosome end-deprotection. The problem is solved by the action of telomerase, a ribonucleoprotein (RNP) enzyme that minimally contains a catalytic telomerase reverse transcriptase (TERT) protein and a long noncoding telomerase RNA (TR) component (Greider and Blackburn, 1987). Telomerase continually replenishes DNA termini by reiteratively copying a templating domain within its integral TR subunit to synthesize telomeric repeats de novo. By maintaining a sufficient length of telomeric DNA on chromosome ends, telomerase ensures that the terminus will be properly sequestered.

Failure to sustain telomere tracts due to telomerase inactivation or mutation of shelterin or CST has devasting consequences. Indeed, a number of "telomeropathies" have emerged as stem-cell diseases and cancer in humans (Bertuch, 2016). The effects of telomere failure are similarly manifested in plants. *Arabidopsis thaliana* mutations in telomerase constituents trigger an inexorable depletion of telomeric DNA through successive generations (Riha et al., 2000), while disruption of CST causes a rapid and catastrophic loss of telomeric DNA (Surovtseva et al., 2009). Critically shortened telomeres activate both Ataxia-Telangiectasia Mutated (ATM) and Ataxia-Telangiectasia mutated and Rad3-related (ATR) mediated DNA damage response pathways (Amiard et al., 2011; Boltz et al., 2012) that culminate in profound genome instability in the form of end-to-end chromosome fusions, and ultimately meristematic dedifferentiation and growth arrest (Riha et al., 2001).

Perhaps because they are sessile, plants have evolved robust DNA repair mechanisms (Manova and Gruszka, 2015), and a high tolerance to genome instability (Boyko and Kovalchuk, 2011). This resilience is on full display in Arabidopsis mutants devoid of telomerase or proteins implicated in chromosome end protection. Telomere-related mutations that are lethal in mammalian cells and even yeast can be viable in Arabidopsis (Surovtseva et al., 2007, 2009; Leehy et al., 2013). Moreover, after the onset of end-to-end chromosome fusion, plants lacking the telomerase catalytic subunit TERT can survive for up to five more generations in the face of ever-increasing genome instability (Riha et al., 2001). Although transcriptomics data reveal striking differences in the response of plants and mammals to the prolonged absence of telomerase (Amiard et al., 2014), the molecular basis of the extraordinary tolerance to telomere dysfunction displayed by plants is unclear. It may derive from the late emergence of a germline, necessitating a deep investment in maintaining stem cell niches: genomes that fail to meet a quality control standard are culled from the population (Riha et al., 2001; Fulcher and Sablowski, 2009; Watson and Riha, 2011). The exceptional malleability of the Arabidopsis genome may also reflect small gene size and insulation from epigenetic silencing (Bennetzen and Wang, 2018), allowing plants to endure initial rounds of telomere fusion and genome rearrangement. More broadly, plants may simply exhibit a high tolerance to genome assault and rearrangement to enhance genetic diversity and adapt to their ever-changing environmental challenges (Schuermann et al., 2005). Whatever its origin, the capacity of plants to withstand telomere failure, at least in the short term, has opened a unique window for researchers to explore fundamental aspects of telomeres and their maintenance by telomerase.

In this article, we highlight recent discoveries in plant telomere research facilitated by technological advances in nucleic acid biochemistry, the advent of abundant genomic resources, and the ability to harness natural variation across different species. These new findings provide unanticipated insight into telomere maintenance and regulation as well as evolutionary surprises to unite telomere research across the Eukarya domain.

Telomere sequence and structure: new twists and a blunt end

Telomere function relies on both the proper length of the telomeric DNA tract and the integrity of the telomere-binding protein complexes that recognize and bind telomeric DNA, either double- or single stranded. Telomere repeat sequences are generally well-conserved for recognition by telomere-binding proteins. The vertebrate-type telomere repeat TTAGGG is conserved across major eukaryote lineages including animals, basal-branching fungi, and some protists, and is likely the ancestral sequence (Fulneckova et al., 2013). However, deviations of the TTAGGG repeat have emerged in select species within each major lineage. The telomeric DNA sequence synthesized by telomerase is determined by the template sequence of the TR component. In plants, the most common telomere repeat sequence is TTTAGGG, suggesting a transition from the canonical TTAGGG repeat through a single-nucleotide mutation in the template sequence of the ancient plant telomerase. Interestingly, the plant-type TTTAGGG repeat appears to have switched to other repeat sequences multiple times through independent evolutionary events. For example, a group of Asparagales switched to the vertebrate-type TTAGGG repeat (Adams et al., 2001), while *Allium* evolved with a long telomere repeat CTCGGTTATGGG synthesized by telomerase with a long RNA template (Fajkus et al., 2016). Other variant repeat sequences have also been described in select groups of plant species (Peska and Garcia, 2020). To ensure that the chromosome terminus is

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fully protected, any alteration in the telomere DNA repeats requires corresponding mutations in the RNA component of telomerase as well as co-evolution of the telomere-binding proteins (Shakirov et al., 2009). As more plant genomes are analyzed, additional telomere repeat sequences may be uncovered, providing new resources to study the co-evolution of the telomere and telomerase complexes.

The discovery that mammalian telomeres terminate in ring-like structures termed t-loops (Griffith et al., 1999) was soon confirmed in plants (Cesare et al., 2003). T-loops form when a single-stranded 3′-extension on the chromosome terminus (G-overhang) folds back and invades the duplex region of the telomere. Symmetrical or nearly symmetrical 3′-overhangs are created on opposite ends of each chromosome in vertebrates: one arising from lagging strand DNA synthesis when the last RNA primer is removed, and the other when the blunt-ended DNA terminus created by leading strand synthesis undergoes 5′- to 3′-resection. The first hint that the architecture of Angiosperm telomeres might differ from vertebrates came when 3′-G-overhangs were detected on just 50% of the chromosome ends in *Silene latifolia* and Arabidopsis (Riha et al., 2000).

Subsequent experiments revealed that half of the telomeres in Arabidopsis that terminate in a blunt or nearly blunt end are capped by Ku proteins (Kazda et al., 2012; Valuchova et al., 2017). A role for Ku in chromosome end protection was unexpected since Ku, in its capacity as a core component of the nonhomologous end-joining repair pathway, encircles double-stranded DNA to facilitate ligation. The blunt-end telomeres of Ku mutants undergo resection followed by substantial telomerase-mediated elongation (Riha et al., 2002; Gallego et al., 2003; Kazda et al., 2012) to create 3′-G-overhangs on both sides of the chromosome. The CST complex, with its high affinity for single-stranded telomeric DNA (Nugent et al., 1996), now associates with both chromosome ends instead of only the lagging strand telomere. Interestingly, the striking asymmetry of telomeres in Arabidopsis is not universally conserved among plants. Blunt-end telomeres are not found in the moss *Physcomitrium patens* (Kazda et al., 2012; Fojtova et al., 2015), implying that they are a recent innovation in evolution.

Since blunt ends are transiently formed during leading strand replication in other species, stabilization of this processing intermediate by Ku implies some evolutionary advantage for plants. It is conceivable that blunt-end telomeres evolved to afford chromosome ends even greater stability, by fortifying the genome in changing environmental conditions (Nelson and Shippen, 2012). Telomeres bearing G-overhangs are vulnerable to inappropriate processing and recombination when t-loops disassemble to access telomerase and lagging strand replication machinery. Even in the closed sequestered conformation, G-overhang-bearing telomeres engaged in t-loops face the risk of telomere rapid deletion (TRD), the precipitous loss of large sections of the telomere arising from the resolution of the Holiday-like junction at the base of the t-loop. First described in budding yeast (*Saccharomyces cerevisiae*; Li and Lustig, 1996), TRD is evident in the overly long telomeres of Arabidopsis Ku mutants (Watson and Shippen, 2007). TRD also occurs in mutants lacking the TEN1 or CTC1 components of CST (Lee et al., 2016); in response to heat shock, telomeres rapidly and dramatically shorten. A variety of biotic and abiotic stresses, including elevated temperature, are proposed to increase the frequency of somatic recombination (Lebel et al., 1993; Kovalchuk et al., 2003) and thus the potential for TRD. The presence of blunt-end telomeres on half of the chromosome ends in plants would decrease the risk of telomere truncation in adverse environmental conditions. Conversely, for mammals, the dynamic nature of G-overhangs may be beneficial because, in conjunction with replicative senescence, it provides an opportunity for elimination of cells with destabilized telomeres. This is a potent weapon in the battle against metastatic cancer (Nelson and Shippen, 2012).

Plant telomere proteins—missing and new links in the evolutionary chain

Telomere proteins evolve rapidly (Linger and Price, 2009; Lue, 2010; Saint-Leandre and Levine, 2020). The trimeric CST is found in plants, budding yeast, and verterbrates, but interestingly a protein homologous to the largest subunit CTC1/Cdc13 has not been identified in fission yeast (*Schizosaccharomyces pombe*). Similarly, vertebrate shelterin consists of a six member complex containing both double- and single-stranded telomere binding proteins (de Lange, 2018), but only one shelterin component, Rap1, appears to have a homolog in budding yeast. While recent structural analyses of shelterin from fission yeast and vertebrates are beginning to provide clues about the origin of this complex (Giraud-Panis et al., 2018), the search for bona fide components of plant shelterin has lagged. A multitude of telomere-associated proteins has been identified in plants (Schrumpfova et al., 2016). Several of these not only bear a single Myb domain with a telobox motif structurally similar to the vertebrate shelterin components TRF1 and TRF2 proteins (Zellinger and Riha, 2007), but also bind telomeric DNA in vitro (Marian et al., 2003; Karamysheva et al., 2004; Kuchar and Fajkus, 2004; Mozgova et al., 2008). Nevertheless, simultaneous disruption of six of the leading TRF-like candidates from *A. thaliana* failed to reveal phenotypes associated with dysfunction of telomere length homeostasis or end protection (Fulcher and Riha, 2015). Hence, the functional equivalent of plant shelterin remains elusive.

Gene duplication and neo-functionalization have profoundly shaped the landscape of interactions and regulation at the chromosome ends in plant genomes (Linger and Price, 2009; Lue and Chan, 2013). A striking example is seen with Protection of Telomeres 1 (POT1), the most highly conserved constituent of the shelterin complex (Barbero Barcenilla and Shippen, 2019). In vertebrates and fission yeasts, POT1 binds the 3′-G-overhang and is essential for both chromosome end

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protection and telomerase regulation. Most organisms harbor only a single *POT1* gene, but in the few examples of *POT1* duplication, the paralogs invariably exhibit functional divergence or sub-functionalization. For example, in *Tetrahymena thermophila*, one POT1 paralog is needed for telomere length maintenance and checkpoint repression, while the other functions in developmentally programmed genome reorganization (Jacob et al., 2007; Cranert et al., 2014). Notably, worms (*Caenorhabditis elegans*) are predicted to harbor four putative POT1 paralogs; one of these binds the G-rich telomeric DNA strand and another the complementary C-rich strand (Raices et al., 2008).

Although the plant kingdom is replete with large gene families, the *POT1* genes in most species are single-copy genes, including the *P. patens POT1* gene, which retains the ancestral functions of chromosome end protection and telomere length regulation (Shakirov et al., 2010). At least two independent *POT1* gene duplication events occurred in angiosperms, one in the Panicoidea and one in the Brassicaceae which includes Arabidopsis (Beilstein et al., 2015). *Arabidopsis thaliana* bears three *POT1* genes, *AtPOT1a*, *AtPOT1b*, and *AtPOT1c* (Shakirov et al., 2005; Rossignol et al., 2007; Kobayashi et al., 2019). *AtPOT1c* is a nonfunctional gene that was silenced soon after it emerged (Kobayashi et al., 2019). The remaining paralogs, *AtPOT1a* and *AtPOT1b*, are the most divergent of any *POT1* paralogs reported to date and exhibit only 52% amino acid sequence similarity. AtPOT1a binds telomeric DNA in vitro, but unlike PpPOT1 it is not essential for chromosome end protection. Instead, AtPOT1a accumulates at telomeres only transiently, likely as part of the telomerase RNP (Surovtseva et al., 2007; Song et al., 2021), where it serves to stimulate telomerase repeat addition processivity (Renfrew et al., 2014). Evolutionary analysis reveals rapid divergence between the POT1a and POT1b lineages (Beilstein et al., 2015). Neither *AtPOT1b* nor single-copy *POT1* genes from related plant species complement an *AtPOT1a* deficiency. Indeed, *POT1a* genes are under positive selection and these changes enhance POT1 binding with CST, presumably to promote telomerase engagement with the telomere (Beilstein et al., 2015). Thus, positive selection of POT1a does not lead to a new function, but rather reinforces an ancient interaction as POT1 binding to CST is also observed in mammals (Wu et al., 2012).

Less is known about AtPOT1b. Ectopic expression of a dominant-negative allele of *AtPOT1b* triggers massive telomere degradation and end-to-end chromosome fusion (Shakirov et al., 2005), suggesting that AtPOT1b may function in a pathway required for chromosome end protection. Strikingly, however, a single amino acid substitution in the nucleic acid binding pocket of AtPOT1b abolishes telomeric DNA binding (Arora et al., 2016). In addition, while POT1a is ubiquitously expressed and cell cycle regulated (Surovtseva et al., 2007), *AtPOT1b* expression is developmentally regulated and restricted to root tips, seeds, and flowers. AtPOT1b, therefore, does not exhibit the properties expected for a canonical chromosome end-protection factor, which would be required for all cells.

AtPOT1b transcription is induced in response to stress, particularly oxidative stress (Huang et al., 2019; Sewelam et al., 2020; Zandalinas et al., 2021). A growing body of literature implicates telomere-associated proteins in the oxidative stress response. DNA is oxidized when the levels of reactive oxygen species (ROS) are too high. If not removed, oxidized bases can trigger global replication fork stalling as well as telomerase inhibition and telomere shortening (Barnes et al., 2019). Because of their high guanine and thymine content, telomeres have been proposed as hotspots for accumulation of 8-oxo guanine (8-oxoG) and thymine glycol (Tg) modifications (Ahmed and Lingner, 2018). A new quantitative assay to measure telomeric 8-oxoG in Arabidopsis provides strong support for this hypothesis, showing telomeres accumulate 8-oxoG at levels 100 times greater than the rest of the genome, and are the primary target upon oxidative stress (Castillo-González et al., 2022).

Like AtPOT1b, vertebrate POT1 is induced in response to ROS (Wang et al., 2018). hPOT1 is among several shelterin components that can stimulate base excision repair (BER) (Miller et al., 2012), the primary pathway for removing 8-oxoG from DNA. Interestingly, since DNA oxidation inhibits telomeric DNA binding by shelterin, the transient disengagement of POT1 from oxidized DNA may operate in a feedback loop to facilitate removal of the damaged telomeric chromatin by BER (Opresko et al., 2005). Telomerase is also responsive to oxidative stress. Human TERT traffics to mitochondria in response to oxidative damage (Santos et al., 2006) and recent data indicate that mitochondrial-targeted damage specifically triggers telomere dysfunction, implying retrograde signaling between mitochondria and telomeres (Qian et al., 2019). A mitochondrial localization signal also resides on the plant TERT protein, but its function remains to be determined. Because ROS plays a crucial role in cell signaling during plant development and in the response to biotic and abiotic assaults, understanding how plant telomeres and telomerase engage the oxidative stress response may have important biological implications.

The convoluted discovery of plant TR

Although the telomerase enzyme was discovered in the ciliate Tetrahymena almost four decades ago, there remains much to learn about this complex, particularly in plants. In contrast to the highly conserved catalytic subunit TERT, TRs exhibit extreme diversity in size, sequence, and structure (Podlevsky and Chen, 2016). TR folds into highly specialized structural domains including two core domains essential for telomerase catalytic function: a template-adjacent pseudoknot (PK) domain and a template-distal helical domain, which facilitate TR-TERT assembly and enzymatic function, as well as additional structural elements that provide a scaffold for binding a myriad of kingdom- or group-specific accessory proteins that enable RNP biogenesis in vivo.

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Due to its remarkable sequence diversity, identification of TR from a new kingdom or new group of eukaryotic species is challenging and often unsuccessful. Although TRs have been independently identified in ciliates, yeasts, and vertebrates decades ago, discovering the TR component from the plant kingdom has been a long and difficult journey. The first putative plant TR molecule was reported in 2011 as an Arabidopsis TERT-bound RNA (Cifuentes-Rojas et al., 2011). Yet, nearly 10 years later, a re-examination of this locus using a clustered regularly interspaced short palindromic repeats (CRISPR)-edited mutant that disrupted the putative TR template did not result in telomere shortening, casting doubt on this RNA as a bona fide plant TR (Dew-Budd et al., 2020). At the same time, two independent studies employing distinct strategies successfully identified the true plant TR as well as a multitude of homologs that show sequence conservation across many lineages of plant species (Fajkus et al., 2019; Song et al., 2019).

The first study leveraged the unusually long 12-nt telomeric DNA repeat sequence CTCGGTTATGGG in *Allium*, which predicts an extended TR template sequence complementary to 1.5 to 2 copies of this repeat sequence. This strategy allowed for effective computational identification of putative *Allium* TR sequences in the *Allium* transcriptome databases (Fajkus et al., 2019). Homologs of the *Allium* TR were found in several groups of plants that have different types of telomeric repeats and the corresponding template sequences conserved in the TR, supporting this RNA as the bona fide plant TR. In addition, the TRs identified from several plant species reconstituted telomerase activity with their corresponding TERT protein in vitro. The disruption of Arabidopsis TR led to telomere shortening, further supporting the identification of the genuine TR in plants (Fajkus et al., 2019).

A second study employed a classical biochemical approach to purify Arabidopsis telomerase holoenzyme. RNA molecules copurifying with the active telomerase holoenzyme (tagged TERT) were identified by next-generation RNA sequencing, which revealed a single RNA present only in the wild-type sample, but not the *TERT* knockout control (Song et al., 2019). Deletion of this RNA in vivo led to telomere shortening and the lack of detectable telomerase activity, which indicate an essential role of this RNA in telomerase function. In vitro reconstitution of this RNA and Arabidopsis TERT protein confirmed an RNA template-mediated processive DNA repeat synthesis. Interestingly, Arabidopsis TR (AtTR) was originally characterized as a hypoxia-responsive long noncoding RNA (Wu et al., 2012). Thus, like human TR (Cheng et al., 2018) and TERT (Santos et al., 2006), Arabidopsis TR is linked to oxygen metabolism, suggesting a cross-kingdom conserved mechanism for oxidative stress responses.

With the true TR from Arabidopsis in hand, secondary structure models of plant TRs were then determined by phylogenetic comparative analysis (Song et al., 2019). The phylogenetic approach allowed inference of a conserved secondary structure model from the alignment of plant TR sequences identified from major clades of land plant species including angiosperms, gymnosperms, and lycophytes (Song et al., 2019). The computational identification of divergent TR sequences from distantly related groups of plant species relied on the use of a structure-based sequence-weigh matrix together with screening of permuted TR template sequences (Song et al., 2019). The conserved TR secondary structure model was further validated by in vivo chemical probing and functional analysis of individual base-paired regions (Song et al., 2019). TRs from these three plant lineages were shown to share a conserved core structure consisting of a template-adjacent PK core domain similar to ciliate and animal TRs, and a template-distal helical domain P4/5/6 crucial for telomerase activity, similar to the helix IV and CR4/5 of ciliate and animal TRs, respectively (Figure 1). Strikingly, the requirement for two TR structural domains is conserved across all eukaryotes including the most basal protist trypanosome (Podlevsky et al., 2016).

A further computational search of *TR* genes by including conserved elements in the TR promoter region successfully identified additional TRs in more early-branching land plants and some algal species (Fajkus et al., 2021). The plant and algal TRs share a similar secondary structure at least in the PK core domain, indicating the PK structure is universally conserved in all eukaryote lineages except for the trypanosome TR which lacks a PK structure in the template-adjacent domain (Podlevsky et al., 2016). The presence of the PK structure in plant TR confirms the functional importance and ancestral feature of PK structure throughout most eukaryotic kingdoms (Figure 1). The lack of a vertebrate/fungal CR4/5 domain in plant and ciliate TR suggests an independent emergence of the CR4/5 domain later along the animal/fungal lineage (Figure 1). While elucidation of plant TR structure begins to close the gaps in understanding the divergent evolution of TR structures, future studies of telomerase in basal lineages of eukaryotes are required to understand the dawn of TR evolution and function.

Filling in a crucial piece in the puzzle of telomerase diversity

Intensive study of telomerase in ciliates, fungi, and animals reveals that like TR, the telomerase RNP complex is remarkably diverse in size, protein composition, and biogenesis (Podlevsky and Chen, 2016). This diversity mainly stems from the unusual variation in TR size and the mechanism of transcription of TR among different groups of eukaryotic species. The small ciliate TRs are transcribed by RNA polymerase III (Pol III), while the large fungal and animal TRs are transcribed by RNA Pol II. The large TRs fold into complex secondary structures consisting of specific structural domains for binding of a myriad of accessory proteins unique to each group of eukaryotes. Interestingly, the TR from the basal eukaryote trypanosome appears to be transcribed by RNA Pol II (Gupta et al., 2013; Sandhu et al., 2013), which supports a "Pol II first" hypothesis for the early

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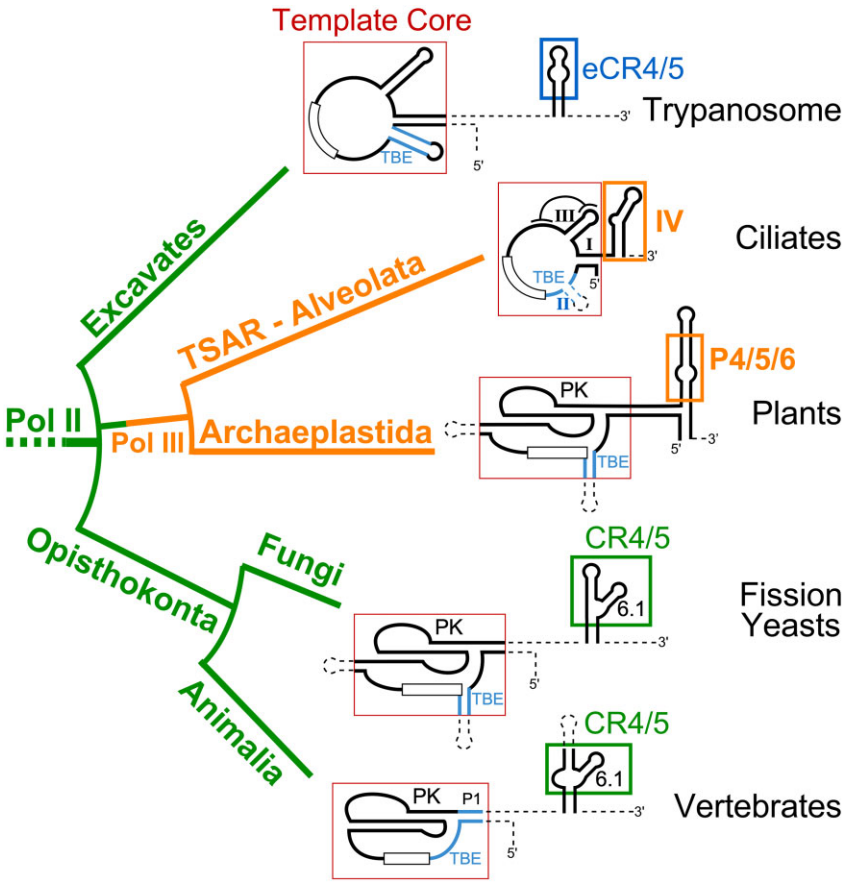

Figure 1 Divergent evolution of TR structure and transcription machinery. The simplified phylogenetic tree shows a possible switch of TR transcription machinery from the ancestral and common RNA Pol II to the RNA Pol III before the branching of ciliates (belong to TSAR supergroup) and plants (belong to Archaeplastida supergroup). The secondary structure of TRs shows the template core domain that includes a template boundary element (TBE) and an essential pseudoknot (PK) structure. The CR4/5 domain is conserved in vertebrates and basal branching fungal lineages. Ciliate and plant TRs lack CR4/5 but contain helix IV or helices P4/5/6 as a second TR domain required for telomerase function.

evolution of TR transcription, that is, TRs in primitive eukaryotes originated as RNA pol II transcripts and the TR transcription machinery later switched to Pol III specifically along the lineage of ciliates as a single evolutionary event. The discovery of plant and algal TRs as Pol III transcripts (Song et al., 2019; Fajkus et al., 2021) suggests that the evolutionary switch of TR transcription from Pol II to Pol III may have occurred surprisingly early before the separation of the two supergroups, TSAR (Alveolates) and Archaeplastida, to which ciliates and plants belong, respectively (Figure 1). Multigene phylogenetic analyses have suggested that the TSAR (Alveolates) and Archaeplastida supergroups are more closely related than the supergroup Opisthokonts that consists of both animals and fungi (Cavalier-Smith et al., 2014; Burki et al., 2020). This is consistent with the "Pol II first" hypothesis of TR transcription evolution and that an early Pol II-to-Pol III switch occurred in the common ancestor of ciliates and plants (Figure 1). However, we cannot fully dismiss other less plausible models such as a "Pol III first" model that require multiple independent switching events in Excavate (Trypanosome) and Opisthokonta (animals and fungi) lineages (Figure 1).

While plant and ciliate TRs are both synthesized by RNA pol III, the plant TRs are larger than ciliate TRs and contain additional structural domains presumably for binding additional accessory proteins. The search for core and accessory proteins in the plant telomerase is ongoing. Fulneckova et al. report that a large number of proteins engage Arabidopsis telomerase (Fulneckova et al., 2021). Recent studies indicate that POT1a and the snoRNA-associated dyskerin complex are associated with the plant telomerase in vitro and in vivo (Kannan et al., 2008; Fajkus et al., 2019; Song et al., 2021). This observation is puzzling as vertebrate dyskerin associates with H/ACA snoRNAs transcribed by Pol II, most notably hTR (Tseng et al., 2015; Logeswaran et al., 2021). The mystery deepens as Arabidopsis dyskerin was shown to engage AtTR via a novel plant-specific three-way junction, and not a canonical snoRNA H/ACA motif (Song et al., 2021). Thus, it is not clear if dyskerin was a component of the ancient telomerase RNP and was lost multiple times in trypanosomes, ciliate, and fungi, or whether dyskerin was gained as a telomerase accessory protein independently in plant and animal lineages. Future studies of telomerase in more basal eukaryotes will undoubtedly shed

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light on the early evolution of TR transcription mechanisms and telomerase RNP composition.

The unusual diversity of TR promises to provide rich information for phylogenetic analysis with a resolution even higher than traditional analysis using the highly conserved ribosomal RNA (rRNA). In addition to TR, the composition of the plant telomerase RNP is likely to be divergent with plant-specific telomerase accessory proteins, distinct from the snRNA-binding proteins in the fungal telomerase and the snoRNA-binding proteins in the animal telomerase. Understanding how the telomerase RNP evolved to acquire and retain these diverse sets of RNA-binding proteins throughout eukaryotic evolution is a fascinating frontier.

Finding the right balance: telomere length control

Cellular senescence is a well-established consequence of unabated telomere shortening in humans (Wright and Shay, 2005). On the other hand, unrestricted telomerase activity can result in hyper-elongation of telomeres, which also has detrimental outcomes, including a higher incidence of cancer in mammals (Blasco, 2005; Adam et al., 2017). Telomere length homeostasis is established and maintained at a species-specific set point determined by a fine balance of forces that extend (telomerase and recombination) and shorten (end replication problem, TRD, nucleases) the telomere tract. The natural telomere length setpoint varies widely between and within different species, including plants (Burr et al., 1992; Fajkus et al., 1995; Shakirov and Shippen, 2004). Within each species, extreme telomere length phenotypes are selected against, while intermediate lengths are favored.

Critical telomere length thresholds that govern chromosome stability and organismal response to stress have been defined in Arabidopsis (Figure 2). In the wild-type Col-0 accession, telomeres are typically maintained in the 2.5–3 kb range (Shakirov and Shippen, 2004). Mutations that result in mild telomere shortening or reestablish a new shorter telomere length equilibrium of ~1.5 kb (NUCLEOLAR PROTEIN 2 (NOP2A)/OLIGOCELLULA 2 (OLI2), DNA replication and repair mutants, transposon control mutants) do not impact chromosome stability (Xie and Shippen, 2018; Abdulkina et al., 2019; Bose et al., 2020). However, mutations that cause the equilibrium to shift below 1 kb or above 5 kb result in the formation of meta-stable telomeres that lose some of their protective functions. Specifically, critically shortened telomeres can result from telomerase inactivation (end-replication problem) or acute loss of CST, leading to massive nucleolytic attack (end-protection problem). These very short telomeres face an increased probability of being recruited into end-to-end chromosome fusions (Heacock et al., 2004; Surovtseva et al., 2009).

Conversely, in the absence of Ku70 or Ku80, Arabidopsis telomerase generates ultra-long telomeres (Riha et al., 2002), presumably because it now has unfettered access to both ends of the chromosome. As with very short telomeres, ultra-long telomeres are unstable and undergo stochastic telomere trimming (TRD) and the formation of extra chromosomal telomeric circles (Watson and Shippen, 2007). The minimal functional length of an Arabidopsis telomere is 400 bp. Telomeres cannot be detected below this threshold, likely because cells harboring such telomeres are inviable (Heacock et al., 2004; Watson et al., 2021). Interestingly, the 400-bp limit corresponds to the minimal length of DNA needed to form a t-loop (Watson et al., 2021), supporting the conclusion that t-loops are essential for genome integrity.

Mechanisms that establish telomere length set point are under intense investigation. Analysis of 653 *A. thaliana* accessions indicates that mean telomere length variation ranges over 10-fold (Fulcher and Riha, 2015; Choi et al., 2021), but our current understanding of the genetic, ecological, and evolutionary bases of such telomere length variations is limited. Association studies and quantitative trait loci (QTL) analyses reveal that telomere length is a quantitative trait, controlled by many genes (Ding et al., 2004; Levy et al., 2010; Codd et al., 2013; Abdulkina et al., 2019), and this observation is consistent with ongoing evolution of this trait. Although systematic deletion screens implicate many genes in telomere length maintenance (Askree et al., 2004; Gatbonton et al., 2006; Liu et al., 2010), the relative contribution of each gene is unclear.

Genome-wide association studies (GWAS) can provide important clues into genes underlying standing genetic variation in telomere length. Common genetic variants at a few loci in animals have shown a replicated association with mean telomere length, including telomerase components TERT and TERC (TR), and the RTEL1 helicase (Ding et al., 2004; Codd et al., 2010; Levy et al., 2010; Bojesen et al., 2013; Codd et al., 2013; Cook et al., 2016Ding *et al.*, 2004; Levy *et al.*, 2010). However, most of the identified variants explain only 1% or less of telomere length polymorphism, implying that the power of natural telomere length variation analysis is still grossly underutilized.

Because of their advanced genomic and mapping resources and the capacity to test the functions and interactions of identified genes, plants present an unparalleled opportunity to analyze natural telomere length variation and to discover causal genes establishing genotype-specific telomere length polymorphism (Koornneef et al., 2004; Shakirov and Shippen, 2004; Maillet et al., 2006). Telomere length in maize genotypes varies by >25-fold (Burr et al., 1992), with genotype-to-genotype variation in this species resembling the whole spectrum of known variation associated with the entire plant kingdom (Brown et al., 2011). A QTL analysis in a maize mapping population uncovered nine QTL for mean telomere length that collectively explained over 30% of the phenotypic variation for telomere length (Brown et al., 2011).

A wide range of genotype-specific telomere lengths has also been uncovered in Arabidopsis accessions (Shakirov and Shippen, 2004; Fulcher and Riha, 2015), confirming that QTL

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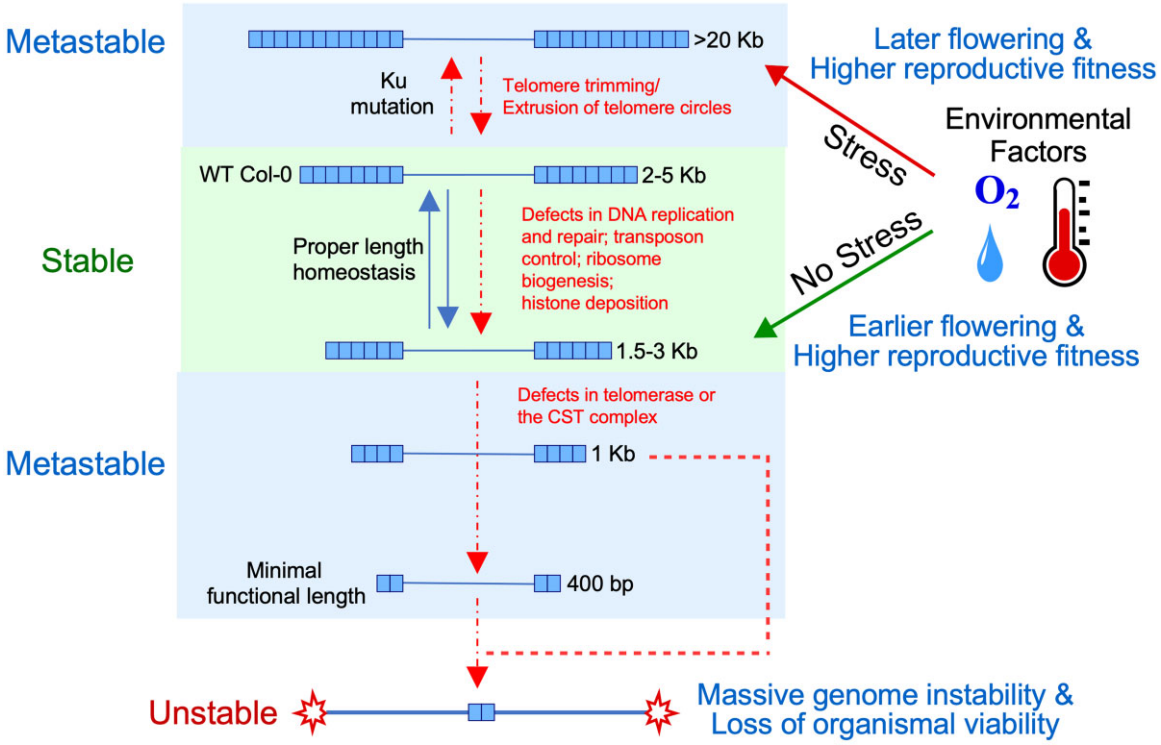

Figure 2 In the Col-0 accession of *A. thaliana*, telomere length is usually restricted to 2.5–3 kb and is generally stable in the 1.5–5 kb range. Mutations that establish length equilibria <1 kb or >5 kb result in the formation of meta-stable telomeres that lose some of their protective functions. Specifically, overly long telomeres observed in *ku* mutants are subject to TRD and production of extrachromosomal telomere circles. Conversely, in the absence of telomerase or components of CST, telomeres shorten. Below 1 kb they begin to be recruited into chromosome end-joining reactions. Continued shortening occurs until they reach a minimum functional length threshold of 400 bp, below which all telomeres become fused triggering massive genome instability. The effect of telomere length homeostasis on plant fitness is mediated by the environment. Under normal growth conditions (no stress), plants of Col-0 genotype flower faster and produce significantly more seeds than the long telomere *ku70* mutants. In contrast, later-flowering *ku70* mutants display higher reproductive fitness in high heat stress conditions, implicating telomere length in life history tradeoffs for flowering time and fitness.

and GWAS mapping represent a promising route to identify causal loci responsible for the standing genetic diversity in telomere length. QTL mapping with Arabidopsis recombinant inbred line populations and a GWAS study resulted in the identification of several candidate regions for telomere length control that mapped to different chromosomes, including one significant single nucleotide polymorphism (SNP) located inside the *TERT* gene (Fulcher and Riha, 2015; Choi et al., 2021). Accessions with the minor *TERT* haplotype B (frequency = 17%) had longer telomeres than haplotype A accessions (Choi et al., 2021). Intriguingly, population genomic analysis provided evidence for a selective sweep at the TERT region, suggesting that the *TERT* gene in Arabidopsis is rapidly evolving. *TERT* also seems to be under selection in other organisms: multiple independent variants at the *TERT* locus were associated with both telomere length and risks of breast and ovarian cancer (Bojesen et al., 2013).

A different QTL mapping study with mean telomere length data for 480 Multi-parent Advanced Generation Inter-Cross (MAGIC) lines identified a major effect QTL interval on chromosome 5. When taken in isolation, allelic differences at this QTL explain 42.2% of the total mean telomere length variation present in the MAGIC population (Abdulkina et al., 2019). Consequently, the gene *NOP2A/OLI2* was identified as the main candidate behind this QTL. Null mutations in *AtNOP2A* cause a 27% decline in telomere length compared to wild-type and ultimately establish a new telomere length set point at this shorter length that is stable over multiple generations. The single-copy human *NOP2* gene encodes a highly conserved 25S rRNA methyltransferase necessary for proper ribosome assembly (de Beus et al., 1994). Notably, human NOP2 associates with telomerase and through direct contacts with TERT stimulates transcription of cyclin D1 (Hong et al., 2016).

Arabidopsis NOP2A/OLI2 interacts with other ribosome biogenesis factors from the OLIGOCELLULA (OLI) pathway, including RPL5A/OLI5 and RPL5B/OLI7 (Fujikura et al., 2009). *RPL5A/OLI5* and *RPL5B/OLI7* paralogs encode highly conserved variants of ribosomal protein 5, which participates in assembly and trafficking of the large 60S ribosomal subunit, and their inactivation in Arabidopsis also lead to shorter telomeres (Abdulkina et al., 2019). The discovery that all Arabidopsis *OLI* genes make important contributions to both ribosome biogenesis and telomere length regulation underscores the enigmatic connection between ribosome

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biogenesis pathway and telomere length homeostasis. Intriguingly, the pleiotropic effects of mutations in several human rRNA maturation proteins, Dyskerin and NOP10, may account for some similarities between diseases of telomeropathies and ribosomopathies (Walne et al., 2007; Orsolic et al., 2016). Thus, telomeres may serve as a hub for a functional crosstalk between multiple cellular pathways with pleiotropic functions (i.e. ribosome biology and rRNA maturation). Disentangling such interactions from telomere-specific pathways is needed to better understand the roles that many genes discovered through genome-wide assays play in telomere biology.

Telomere connections to fitness, stress response, and cell physiology

The presence of substantial (and still largely unexplained) telomere length variation in natural populations of Arabidopsis and many other eukaryotic species suggests that natural selection is highly complex, due to myriad pleiotropic relationships or balanced polymorphism (where there is some spatial or temporally varying selection) that keeps multiple alleles alive. In conjunction with emerging studies revealing connections between telomere maintenance machinery and stress (Epel et al., 2004; Garrett-Bakelman et al., 2019; Hachmo et al., 2020), a relationship between telomere length and reproductive success and lifespan has been discovered in birds and other animals (Haussmann et al., 2005; Bauch et al., 2014; Asghar et al., 2015). Therefore, it seems likely that environmental stress will also impact plant telomeres. Additionally, associations between telomere length, geographic origin, and climates have been reported in Arabidopsis, rice (*Oryza sativa*), and maize (Choi et al., 2021), supporting the notion that environment may play a key role in the maintenance of natural telomere length polymorphism within species.

The study of telomeres in the context of ecology and evolution represents a new frontier with exciting opportunities for identification of factors affecting both telomere length and adaptation to the environmental conditions or stress (Monaghan and Haussmann, 2006; Bize et al., 2009). A recent study evaluating how stress impacts plants bearing telomeres of different lengths revealed that Arabidopsis genotypes with short and medium telomere length exhibit little telomere length plasticity (telomere length changes in response to the environment), while genotypes with longer telomeres (*ku70*) undergo substantial shortening in response to a moderate dry treatment (Campitelli et al., 2022). Hence, abnormally long telomeres show significant plasticity and may become fragile under stress leading to resection or deletional recombination. This observation may provide clues for why a specific upper-range limit of telomere length is established in each species or population.

In the same telomere ecology study, several instances of genotype by environment interactions were detected, including for fitness (Campitelli et al., 2022). Specifically, long telomere *ku70* mutants showed higher reproductive fitness in a high heat environment and lower fitness in cooler conditions, indicating that longer telomeres provide selective advantages under temperature stress (Campitelli et al., 2022). Thus, telomere length appears to be an adaptive trait, with the benefits of telomere length change in one environment coming at a cost to performance in alternative conditions (Figure 2). Additionally, significant and widespread pleiotropic effects of telomere length variation on other plant traits were discovered in the long telomere *ku70* mutants, including a strong 3- to 4-day delay in flowering time, a key aspect of life history strategy (Campitelli et al., 2022). Correlation between telomere length and flowering time has also been observed in maize and rice, suggesting that such relationship is widespread and evolutionarily conserved (Choi et al., 2021).

The unanticipated pleiotropic effects of telomere biology on fitness, development, and cell physiology may be explained by inevitable tradeoffs between organismal response to stress or energy constraints and the need to maintain an appropriate telomere length (Giraudeau et al., 2019). Under the Telo-Hormesis hypothesis, stress or changing environmental conditions cause resources to shift away from telomere maintenance to redox signaling and changing gene expression profiles (Jacome Burbano and Gilson, 2021). Indeed, proper telomere length maintenance is likely an energy-consuming process, and telomere homeostasis may represent a compromise between potential gains in organismal survival or fitness and associated inevitable energy costs. Elucidating how life history strategy intersects with natural variation in telomere length maintenance may have broad implications, but defining the "cause and effect" relationships for telomere dynamics and organismal fitness is inherently complex. Pleiotropy in telomere maintenance is widespread and noncanonical (indirect pathways) clearly contribute to telomere regulation (Askree et al., 2004; Gatbonton et al., 2006; Ungar et al., 2009; Abdulkina et al., 2019). A deeper dive into these types of enigmatic relationships for telomeres and telomerase may reveal new mechanisms underlying adaptation and survival of individual organisms and populations.

Concluding thoughts

Plants evolved with new strategies to maintain telomeres and genome stability in changing environmental conditions. The evolution of telomeres and the telomerase enzyme in different plant lineages reflects these novel adaptations. Such adaptations include pleiotropy of important genes that connect telomeres to plant physiology and stress response in unanticipated ways. The remarkable stability of plant telomeres may reflect the multiple modes of telomere length regulation and thus highlight their novel adaptations to a life rooted in the ground.

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